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# IMMUNOFLUORESCENCE IN INFECTIOUS PATHOLOGY

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Credit for having thought of and developed the technique of immunofluorescence goes to Coons [1].

In 1941-1942, this author noted that it is possible to mark the molecules of antibody globulins with a fluorochrome without altering their serological reactivity. In effect, the antibodies thus conjugated become capable of attaching themselves specifically to the corresponding antigens and their fluorescence makes them visible under ultraviolet microscopy.

Thus, Coons was able to visualize for the first time Type III pneumococci antigens using a solution of antibody globulins coupled with fluoresceïn isocyanate and coming from a hyperimmunized rabbit serum [2].

Subsequently, this method was perfected and used widely in bacteriology, virology, parasitology, histo-immunology, both for basic research and for clinical diagnosis.

## The Principles of Immunofluorescence

### Direct Method

Identification of a bacterium on a microscopic preparation containing several species of bacteria allows us to understand the very simple principle of this method.

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After fixing, the preparation is brought in contact with a solution of marked antibodies (also called conjugated) using a fluorochrome. The antibodies corresponding to the germ whose identification is being sought concentrate at the surface of the bacterium while they attach themselves on its "antigenic sites."

The excess of non-fixed antibodies is eliminated by washing, the microscopic examination then proceeds by using a light source whose wavelength has been so selected that the fluorescence of the "marking" fluorochrome is excited at its optimum rate (generally blue-violet, ultraviolet rays).

The bacteria on which the specific marked antibodies are fixed appear to be surrounded by an easily visible fluorescent ring. In turn, the other species of bacteria are not made fluorescent and remain invisible.

Thus schematically illustrated, the technique of immunofluorescence combines the rapidity of a microscopic examination with the immunological specificity of a serological examination. The bacterium made visible by known fluorescent antibodies is at the same time identified by them.

It may happen that the detection applies no longer to a bacterium, but to antigens in the molecular state, located within the tissue or the cells; more or less confluent brilliant points are then observed. Inframicroscopic viral antigens can thus be located in the nucleus of a cell infected with conjugated antibodies, specific to the virus under study.

As described, the direct method only utilizes the antigen and the fluorescent homologous antibody.

Indirect Method

The other variations are headed by the indirect method, also called the "sandwich" method or the "double layer" method [3].

First, the preparation is brought in contact with a non-conjugated antiserum. The antibodies enclosed attach themselves to the antigen and then the unattached excess antibodies are eliminated by washing.

Next, a second antiserum conjugate is added, containing anti-gamma globulin antibodies of the animal species which furnished the first antiserum. This

second layer of fluorescent antibodies attaches itself to the first layer of the non-fluorescent antibodies (with the excess again being eliminated by washing). The first layer is fixed on the antigen.

The first layer consists of gamma globulins which behave like antibodies vis-a-vis the antigen and like antigens vis-a-vis the second layer of fluorescent antibodies.

This indirect method is thus based on Coombs' principle.

It permits identifying an antigen, for example, bacterium A with the aid of a first layer of anti-bacterium A rabbit antiserum, followed by a second layer of conjugated rabbit anti-gamma globulin antiserum.

It also permits, and this is important, identifying an antibody. The first layer, consisting of the serum under study (for example, patient serum) is brought in contact with a known antigen. The second layer encloses the conjugated anti-gamma globulin antibodies of the serum of the first layer (for example, human anti-gamma globulins).

Seen from this angle, the indirect method can be used in serology the same as a serodiagnosis.

There are also other variants of the immunofluorescence technique and they are used less frequently than the preceding ones. For example:

1. The anti-complementary method [4] where a conjugated antiserum makes the complement fixed on an immune complex fluorescent (a principle similar to that of the fixation reaction of the complement).

2. The method of fluorescence inhibition.

## Techniques and Material Used

### 1. Preparations of Marked Antibody Solutions

The first operations are the following:

(1) Hyperimmunization of animals (most frequently rabbits) with known antigens.

(2) The serum is collected sterile and titrated (by agglutination or by complement shifting). A high antibody content is indispensable.

(3) The immune serum is generally fractionated by the ammonium sulfate. We obtain a solution of globulins whose weight content is determined by nitrogen dosage.

Globulin "Marking"

The fluorochromes used [5] are in fact chemical derivatives of common fluorochromes capable of reacting and conjugating with aminated radicals of aminated globulin acids.

(a) Derivatives of Fluorescein

(1) Fluorescein isocyanate was the first fluorochrome (Coons).

(2) Presently, based on the work performed by Riggs and associates [6] we use primarily fluorescein isothiocyanate. It is actually more stable than the preceding compounds.

It combines with the aminated radicals of proteins by its isothio grouping.

(b) There are also other fluorochrome derivatives, particularly Lissamine-Rhodamine [7] and 1-dimethylamino-naphthalene 5-sulphonic acid.

Conjugation is accomplished by cold agitation of the globulin-fluorochrome mixture. The amount of fluorochrome to be introduced is a function of the weight of the globulins which are to be marked. For the fluorescein isothiocyanate the ratio adopted is generally 1 to 20. This is very important because the proportion of fluorochrome molecules marking each antibody molecule must be optimum.

The solution is then dialyzed or passed over a Sephadex column to eliminate the excess fluorochrome not combined with the globulin.

Both these operations must be performed under rigorously determined temperature and pH conditions.

After having performed the necessary "drainage" the marking ratio is verified (F/G) and the final step is dividing the solution of conjugated antibodies in

order to store it in a freezer at -20° C. Under these conditions the solution can be preserved for more than one year.

2. Slide Preparation

If a microbiological sampling is involved (throat sampling, various exudates, stools or of a microbium culture), the spreading over the slide must be thin and homogeneous.

In histology, the sections are prepared frozen to avoid the alteration of the antigens. They are then deposited on the slide. In virology, sections of cellular cultures or tissues are more frequently involved.

In all cases, the slides are fixed by immersion in a mild fixing agent: alcohol, acetone for example (the slides so prepared must not be conserved too long, even when cold).

The preparations are then put in contact with the conjugated antibody solution. This is accomplished by applying to each slide some drops of this solution which is left alone for 20 to 30 minutes. The slide is then mildly rinsed by immersion in buffered physiological water (pH 7) so as to eliminate the excess antibodies which are not fixed on the antigens.

After this rinsing, the preparation is covered with a drop of buffered glycerin (pH 7) and a lamella; it is now ready for microscopic examination.

In the "indirect" method, the preparation is first covered with specific non-conjugated antiserum, then rinsed for the first time. It is then covered with conjugated anti-globulin serum and rinsed the second time. It is then mounted with glycerin and lamella.

3. Microscopic Examination

An ordinary microscope with black background condenser is used. Lighting is very sensitive. In fact, it must be intense and have a wavelength such that it excites the fluorescence of the fluorochromes. The ultraviolet wavelength (or of the blue violet) is generally adequate.

The light source is a mercury vapor lamp. A so-called "exciter" filter placed before it stops the rays of the visible spectrum whose wavelengths are useless

and allows only passage of ultraviolet radiation.

These, upon arriving at the preparation "excite" the fluorochromes which emit their fluorescence. Involved is the fluorescence of a yellow-green color for the fluorescein derivatives, orange-red for those of rhodamine.

At the lens level, a second filter stops the ultraviolet rays and allows the passage of fluorescent wavelengths only.

It is possible to photograph the preparations with highly sensitive film [5, 8, 9].

## Applications

The applications of immunofluorescence vary greatly and are many in number. They have already been the object of many hundreds of publications.

By entering the practical field, we will have primarily in view the assistance which these new techniques can perhaps contribute to the diagnosis of certain infectious diseases. It is not that immunofluorescence is unimportant in the experimental field of fundamental research, far from it; we propose also during the verbal explanation to point out some studies which have benefited considerably, in bacteriology, virology, and immunology, from the immunofluorescent method.

### (A) Practical Applications to the Diagnosis of Bacterial Infections

#### 1. Enteric Infections by Intestinal Germs

(a) In infantile gastroenteritis due to pathogenic colibacilli immunofluorescence has been used for the first time by Whitaker and his associates in 1958 [10].

Their study covers 128 specimens of stools collected during an O 127-B8 colon nursery epidemic. The samplings were examined according to the "direct" method with a conjugated anti-O 127-B8 rabbit serum. The results were compared with those of the classical identification method (culture followed by sero-agglutination).

The correlation between the two techniques was observed in 69% of the cases (of 53 positive and

36 negative samplings), in 30% of the cases the results were positive with the immunofluorescent method alone (excess disagreement).

In 1960, Nelson and Whitaker [11] examined 355 stools of infantile gastroenteritis patients with 10 conjugated rabbit antiserums corresponding to the 10 enteropathogenic serotypes of colon bacilli. They found 98% of correlations with the classical method and a very slight percentage of excess or shortage disagreement.

Cohen et al, in 1961, also found 87% correlation and less than 7% of excess disagreement [12].

Thomason, in 1961 also found 88% correlation and less than 15% disagreement, all by excess [13, 14, 15].

In France, Fournier in the Le Minor Laboratory, as well as Dropsy and Muick [16, 17, 18] used this method. In the vast majority of his observations, Fournier claimed results which agreed with the classical technique, in 8% of the cases there was disagreement by excess.

All these authors agree on attributing to the immunofluorescence method real value. It would seem to be almost as specific as the classical method of culture agglutination. The majority of the authors in fact tested their conjugated serums with a very large number of bacterial species susceptible of being encountered in the stools. They found no more crossed reaction than with the classical serological methods provided, however, the serums were used with optimal dilution.

These very good results recorded in tracking down pathogenic colon bacilli appeared to be enhanced by the presence of a K antigen of the B variety, an envelope antigen surrounding the somatic O antigen.

The rapidity and simplicity of the examination also accentuate the advantages of immunofluorescence. About one hour after the sampling, the result can be obtained; this makes it theoretically possible to identify infants carrying the pathogenic colon bacilli starting with their arrival at the hospital or from the beginning of an epidemic.

We point out that an indirect method, using an agglutinating rabbit serum (commercial) and an anti-rabbit conjugate is also being proposed.

All these acquisitions are evidence that infantile

gastroenteritis with pathogenic colon bacilli is one of the least disputable diagnostic applications of immunofluorescence.

(b) At the opposite end, unfortunately there are the bad results of this technique whenever giving evidence of other enteropathogenic bacteria is involved, such as salmonellas, shigellas, and choleric vibrions.

With these germs, the crossed reactions due to O antigens common to many intestinal germs are in fact so numerous that they render the method useless [19, 20, 21].

2. Whooping Cough

The bacteriological diagnosis of whooping cough by cultures of pharynged samplings over a Bordet and Gengou medium has the double inconvenience of frequent failure and requiring an average of 3 days' time. Thus, direct immunofluorescence was tried for the first time in 1960 by Donaldson, Whitaker and Nelson [22, 23].

These authors report that in 128 cases of clinically confirmed whooping cough, immunofluorescence was positive 100 times while it was negative 120 times against 122 control subjects in good health or with other infections (this makes an excess of two errors).

Kendrick et al [24] in 1961 compared the results of the culture with those of direct immunofluorescence; the 130 pharynged samplings made among whooping cough suspects were transported to the laboratory in a liquid medium. 98 samplings were negative with the two methods, 21 were positive with the two methods, 4 were positive by the culture alone and 7 were positive by immunofluorescence alone. In this last group, the clinical diagnosis of whooping cough was confirmed for no more than half the children; still here, excess errors appear possible.

In France, J. Marie, F. Herzog and M. Gaiffe reported in 1963 the results obtained from 100 samplings [25]. In the 55 cases where clinically confirmed whooping cough was involved, immunofluorescence was 45 times positive, 10 times negative, and the culture was 37 times positive and 18 times negative. In the 45 cases where no whooping cough infections were involved, immunofluorescence was 44 times negative and once positive.

Despite some errors by excess, the crossed reactions with other germs and the delicate management

of the method indicated in the preceding reports, all the authors agreed that direct immunofluorescence is a technique of the future for the diagnosis of whooping cough, particularly at the beginning of the infection (the catarrhal period) if the subjects have not yet received antibiotics.

Our personal experience of the method forces us to be less optimistic, while fully realising that it can be of service.

We noted in fact that an examination of the slides was often difficult due to non-specific fluorescence of certain elements of the smear (desquamated cells, mucous, leucocytes, food left-overs among infants), also because of the rarity and the hardly characteristic morphology of "Bordetella pertussis" which is a very small germ. Furthermore, like Kendrick, we observe a strong crossed reaction with Bordetella para-pertussis and Bordetella bronchi-septica (even after very strong dilution of the conjugated serums).

In an attempt to eliminate the possibility of errors by excess, we paid great attention to the preparation of the serum (passage through Sephadex, correct proportionality of marking, drainages, optimum dilutions) and we observed rigorously the criteria of positivity. This means that in clinically confirmed whooping cough cases, we recorded a considerably lower percentage of positive results than that of the aforementioned authors.

We are thus not very satisfied with the direct immunofluorescent method and we also tried the indirect serological method. It appears to yield good results provided, however, certain precautions are observed in the preparation of the human anti-gamma globulin conjugates; it would only be of interest if the appearance of the antibodies were relatively precocious, which is unfortunately very little likely.

3. Diphtheria

It is known that the direct identification of Corynebacterium diptheriae is impossible by the routine technique of Gram coloration in the smear made by pharynged sampling since the germ is in fact mixed with numerous saprophytes.

It is necessary to use culture. The Kleba-Loeffler bacillus pushes forward in 16 to 18 hours and often it is necessary to characterise it by its

experimental pathogenic capacity with the guinea pig since the differential diagnosis may occur with other saprophyte diphtherimorphous bacteria. A direct identification method by immunofluorescence would thus be very useful.

Whitaker et al [26] "conjugated" a diphtheric antitoxin and based on preliminary studies made with different Coryne bacteria, they assumed that only Coryne bacteria was made specifically fluorescent. Among 9 subjects stricken with diphtheria the results were 8 times positive, while they were negative in a large number of non-diphtheria afflicted subjects. Fluorescence which appeared like a "halo" around the germ was attributed to the secreted toxin.

Lately, other bacteriologists have shown the impossibility of differentiating with conjugated antitoxin the toxigenic strains of Coryne bacterium diphtheriae from non-toxigenic strains. Bacterial antibodies thus appear to be acting at the same time as antitoxin.

This observation led Moody and Jones in 1963 [27] to make the conjugated antimicrobium test, prepared by injecting with great precaution the guinea pig with live strains still provided with thermolabel K surface antigen (antiserum O K) and autoclaved strains (antiserum O).

The best results were obtained with O K antiserums. The various diphtherimorphous Coryne bacteria tested showed no fluorescence except the rare toxinogenic strains of Coryne bacterium ulcerans. The diphtheric bacilli in contrast yielded good fluorescence, but at very low dilutions (1/5). The streptococci and the staphylococci yielded crossed fluorescence. Moreover, it was impossible to distinguish the toxigenic strains from the non-toxigenic strains of Coryne bacterium diphtheriae.

Despite these difficulties, the authors believe that the method could be applied to diagnosing diphtheria, not directly on the throat smear, but on a smear made after several hours of incubation (3 to 6 hours) of the sponge in a liquid medium.

4. Streptococcus Infections

To study streptococci, to determine their groupings, the rapidity of immunofluorescence could make this technique preferable to the Lancefield method.

In 1958, Moody et al prepared conjugated group antiserums with a fluorochrome. They observed a strong crossed reaction between the streptococci of group A and those of group C, a lesser crossed reaction between the streptococci of groups A and G. To extenuate this inconvenience, Moody et al tried to "drain" the anti A serum with streptococci C and G [28].

Other authors [29] proposed another inhibition method consisting of applying a non-conjugated antiserum C followed by a conjugated antiserum A to a first smear; on a second smear identical to the first only conjugated antiserum A is applied. Comparison of the fluorescence of the two scumbles shows an inhibition of the latter on the first scumble if streptococcus C is involved.

Thus, these preliminary efforts showed that with good quality drained antiserums, immunofluorescence could compare favorably with the Lancefield method.

In clinic, the first studies made on pharynged samplings were not demonstrative, either because positive fluorescences were noted when the culture was negative or vice versa. In fact, this is a technique where germ cultures and immunofluorescence are combined which appears to give the results expected.

The sponges are placed in the bath for 2 to 5 hours, within a medium of liquid culture. After agitation, the smears are prepared for immunofluorescence.

With this method Peoples et al [30] obtained 215 positive results (in the same series, 235 were obtained by the traditional method) and 305 negative results (315 negative results by the traditional method.

Other authors also reported their results [31, 32, 33, 34, 35] and as a whole, there is about 75% to 95% agreement between the two methods.

Many divergent results appear essentially due to false positive fluorescences.

It is derived from these early efforts that immunofluorescence is too often untrue now, but that it deserves more profound experimental and clinical study.

5.

Many other applications have been forecast for the diagnosis of various bacterial infections, some of which

are still in the experimental stage. We cite the work performed on staphylococci (those which secrete coagulase could be distinguished from the non-pathogenic types) [36, 37] pasteurellas, brucellas, pseudo-mallei Malleomyces, carbon bacterial infection, leptospiroses, the pork chigger, etc.

We will dwell for a moment on the applications of immunofluorescence to the diagnosis of purulent meningites and venereal diseases.

The direct method has been applied to patients with purulent meningitis of the Pfeiffer bacillus [38]. In 43 samplings the results were almost identical to those of the classical methods (Gram and cultures). However, immunofluorescence has turned out to be more sensitive since it alone permits, in 5 samplings of cephalorachidian liquid, to give evidence of fluorescent bacilli (particularly after antibiotic treatment). Specificity appears to be good, because the preliminary tests only showed a weak crossed reaction with meningococcus strain.

Other authors tested direct immmunofluorescence on meningococcus strains [39] and monocytogenic Listeria [40].

Under the heading of purulent meningites, one should mention that immunofluorescence has some beneficial qualities. In fact, in contrast with what occurs in throat or stool samplings, those of the cephalorachidian liquid are rarely invaded by many microbial species at the same time and the reading of the slides is thus relatively easy. In spite of this, the superiority of immunofluorescence over traditional techniques does not seem to have asserted itself.

For the diagnosis of blennorrhagia, Deacon et al first used the direct immunofluorescence method [41]. They used conjugated antiserums prepared by animal injection of young gonococcus cultures, including a type K antigen; to eliminate the cross reactions with the other Neisseria, the serums were drained with meningococcus strains.

Especially in women, where blennorrhagia easily takes on a subacute and insidious speed, where bacteriological diagnosis is at times difficult, the advent of new techniques deserves attention. Here, Deacon compared the results of direct immunofluorescence of smears and direct immunofluorescence after previous culture of the

samplings for 16 to 20 hours with those of traditional cultures.

Based on the conclusions reached by this author, it appears that the delayed immunofluorescence method yields the same results as the culture, it requires less time, but meanwhile it has neither the speed nor the simplicity of the direct method; unfortunately, and still according to Deacon, it will often be by shortage [42].

On the diagnosis of syphilitic infection, only a few remarks will be made.

An indirect serological method will be used which detects the antibodies by placing the serum of the patient (at a convenient dilution) in contact with a preparation on a treponema slide (Nichols strain) originating from an experimental rabbit orchitis. The antibodies attached to the treponema antigens are then revealed by a human anti-gamma globulin conjugate.

In 1957, Deacon et al used this technique for the first time [43].

In France, it was introduced by Borel and Durel [44] in 1959. Today many serologists have adopted this method.

Theoretically, one would be justified in expecting much from a method which uses the "colorless treponema" itself as an antigen.

However, Pillot and Borel [45] showed that immunofluorescence detected not only specific treponema pallidum antibodies but also the group anti-protein. The possibility of using the Reiter strain (non-pathogenic) as an antigen illustrates further this peculiarity.

Although the reagents do not play a role in immunofluorescence, it is not possible to use this method to control falsely positive reactions with cardiolipin (also very rare) particularly if the serum becomes the complement with the Reiter antigen [46].

The specificity of the method is also reduced by the possibility of positive fluorescence with normal serums; to avoid this disadvantage, the serums examined must be diluted. However, agreement is not obtained on the optimum content to be selected: 1/30, 1/50 for some, 1/200 for Deacon [47, 48].

In fact, it may be difficult to know where non-specific fluorescence and specific fluorescence begin.

Moreover, and on another level, one should also consider the quality proper of the antigenic treponema preparations and of the conjugated serums which are not standardized.

Likewise, in spite of its rapidity, its sensitivity and contrary to the information obtained from certain authors [49], the majority of the authors share the opinion of Mac-Entegard who feels this technique should not be utilized as a routine serological method in the syphilis diagnostic laboratories [5].

At any rate, it should not lead to an abandonment of the traditional methods, well codified, using cardiolipid or Reiter antigens which have already proved themselves. Since the Nelson test is the only sure method, it is the controlling factor in case of doubt.

(B) <u>For P.P.L.O. Infections</u>
(Peri-Pneumonia-like Organism)

We must remember here only the serological diagnosis of primitive atypical pneumonias due to a mycoplasm, the Eaton agent.

First let us recall that these studies by Liu et al [50] showed that it was possible to detect through immunofluorescence the Eaton microplasm at the level of the bronchial tree of infected chicken embryos.

From these studies, the clirical applications, studied by Cook [51] and in France by Thivolet, Sohier et al [52], are derived.

Involved is a serological method of indirect immunofluorescence where the serum of the patient is brought in contact with the antigen represented by sections of experimentally infected chicken embryo lungs; the fixed antibodies are then detected by a human anti-gamma globulin conjugate.

Among the 102 patients with atypical pneumonia, examined by Thivolet and Sohier:

30 had a positive serology for the Eaton agent at a dilution equal to or greater than 1/10 and negative (except 2) for the antigens of influenza, adenovirus, Q fever and ornithosis.

56 had a negative serology vis-a-vis the Eaton agent.

16 had a positive serology for one of the other aforementioned antigens.

The diffusion this method should have will certainly be curbed by the difficulty of preparing antigen slides.

## (C) Viral Infections

Applications to diagnosis are still very limited. They use the direct method:

Liu proposed rapid diagnosis of influenza infection by immunofluorescence of specific viral antigens affecting disquamated cells of throat washings. The percentage of positive cases unfortunately is very small [53].

Immunofluorescence has also been used in studying the herpetic infection [54] and the rapid discovery of poliomyelitis virus in cellular cultures inoculated with patient stools [55].

In rabic infection, immunofluorescence has permitted many authors [56, 57, 58] to recognize viral antigens in the cytoplasm of brain cells or the salivary glands of slaughtered, dead, or experimentally inoculated animals. This method is more sensitive than those which detect the Negri bodies by histological coloration.

## (D) Parasitology and Mycology

In parasitology Goldman [59] showed that immunofluorescence may be used to distinguish different varieties of amoebas provided drained serums are always used.

The same author [60] has accomplished making Toxoplasma gondi fluorescent (direct method) in peritoneal exudates of infected mice. He also detected antibodies in man by using an indirect inhibition method and he did so with results essentially identical to those of the so-called dye test method [61].

Recently, Garin and Ambroise-Thomas [62], using the indirect method, obtained results comparable to those of the lysis test in France based on 189 serums studied.

Let us also record the applications proposed for

detecting trypanosomes, helminths, and Trichomonas vaginalis.

In mycology, the studies were only roughed out in candidoses, histoplasmosis, and cryptococcosis , but the difficulties have already been pointed out. They consist of crossed reactions between pathogenic and non-pathogenic Candida [63]; on the other hand, autofluorescence of mycelium elements is not one of the least obstacles.

## Comments of a Practical Nature

Throughout this study, it was possible to note that the immmunofluorescence method, whose principle is simple and elegant, yields results whose interpretations is frequently delicate since the technique itself offers some difficulties [64, 65, 66].

While it has the advantage of immunological specificity, this method also has its limits. The community of antigens among the various bacteria is the cause of crossed reactions and these are the cause of the early difficulties. Some of these reactions observed in the immunofluorescence are not always predictable by traditional serology, such as, for example, the crossed reaction between streptococci of the groups A and C. It is therefore necessary to investigate these reactions systematically before any application of the method in an as yet unexplored area and before any new conjugated serum.

Indeed, this difficulty can be attenuated by "draining" the antiserum with antigens which yield these crossed reactions. An effort is also being made to use maximal dilution of the conjugate which still yields specific fluorescence (observed only for lower strength levels). In spite of these precautions, it must be agreed that under some circumstances, (salmonellas, shigellas, vibrions ) the antigen communities are so extended that they force a renunciation from the method. Another obstacle is that of non-specific fluorescence.

These are due either to autofluorescence of certain substances (this is particularly the case of vegetable substances) or, what is more bothersome, to the diffused background fluorescence of the preparation and to the non-specific fluorescence of the antigen.

The latter are apparently produced by the existence of numerous "natural" antibodies in the serums (vis-a-vis the tissue elements, bacteria and cells) and

by the tendency of the proteins of the antiserum to attach themselves to a large variety of substances.

Regardless of the mechanism, these fluorescences make the interpretation of the results very difficult (whooping cough, syphilis...). To eliminate them, the following means are used:

1. Dilution of serums;

2. Draining them with pulverized desiccated organs (Coons);

3. Specific treatment of antigenic preparations (formol, alkaline buffers);

4. Mainly optimum marking.

In fact, as shown by Coons and Goldstein[67, 68, 69] the importance of these non-specific fluorescences appears to be partly a function of the degree of conjugation, i.e. of the number of fluorochrome molecules attached to each antibody molecule. The fluorochrome/globulin ratio which appears to be most suitable is approximately 2 to 1. A lower marking reduces the sensitivity, a higher marking attenuates the specificity.

The difficulties foreseen are generally a source of errors by excess. Errors by shortage are also possible, for example, if a reduction in the reactivity of the conjugated serums or of the antigen preparation comes into play. To avoid these errors, systematic controls must be established.

On a very technical level, some difficulties of the immunofluorescence method should be pointed out:

1. The apparatus and the fluorochromes are relatively costly;

2. Conjugation of serums continues to be a delicate operation. Since commercial serums are not always excellent in quality, they are, at least, not always standardized;

3. Certain antigens (such as Eaton agent ) are difficult to prepare. Although rapid, the method which requires many manipulations offers only a very low yield (particularly if the indirect technique is involved which takes twice as long as the direct technique).

Lastly, eye fatigue, which develops rapidly, prevents the examination of too many slides per session.

Based on this, it is necessary to view individual variations in appreciation of the intensity of fluorescence which makes the scales adopted rather arbitrary.

In conclusion, immunofluorescence retains a very lively interest in spite of all the difficulties just mentioned.

In the area of experimental research, particularly immunology and virology, the very fruitful results already provided by immunofluorescence will continue to expand.

In the practical area, designed to provide a diagnosis of infectious diseases, its applications are still in the rough stage.

Prior to a greater diffusion of the method, it would be desirable to specialize the various applications in well equipped centers which have perfectly standardized reactants.

At the end of this study we would like to offer our appraisal by quoting Mac Entegart [7]:

"Many opinions expressed on the role that immunofluorescence may play in bacteriological diagnosis seem too optimistic. The value of the method for the rapid identification of certain bacteria is indisputable, but the use of the fluorescent antibody technique requires no less qualification, experience and judgment than any other bacteriological method and the impression that it could lead to a 'push button' method of diagnosis in bacteriology is no doubt deceptive."

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